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Comparison of the Effects of Acid and Bile in the Canine Esophageal Mucosa during Acute Experimental Reflux Esophagitis

Ph.D. Thesis

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1. INTRODUCTION

1.1. GASTRO-ESOPHAGEAL REFLUX DISEASE

Repeated regurgitation of the gastric/duodenal content to the esophagus may lead to gastro-esophageal reflux disease (GERD) which is responsible for a high proportion of dyspeptic/digestive symptoms in the European population.

The leading symptoms are heartburn, retrosternal pain, dysphagia or odynophagia. Several mechanisms are involved in the pathogenesis including lower esophageal sphincter (LES) incompetency, decreased esophageal clearance, weak esophagus body motility, abnormal gastric emptying, hiatal hernia, medications that weaken LES, anticholinerg drugs, calcium-channel blocking agents. Prolonged regurgitation of the stomach or duodenal content can lead to different complications, such as esophagitis, ulcer, stricture, and Barrett's esophagus, which is predisposed to malignancy. Barrett's metaplasia develops when the normal squamous epithelium is replaced by a columnar-lined, metaplastic epithelium, and this condition occurs in 15% to 20% of GERD patients. The prevalence of adenocarcinoma of the esophagus and esophagogastric junction is increasing at an extraordinary rate.

In the last decades GERD became the most frequent inflammatory disease of the foregut in the Western world (Gallup 1988). The diagnostic tools for appropriate diagnosis are esophageal endoscopy and biopsy, manometry, 24 hours pH-metry, and optionally X-ray and/or Bilitec examination.

1.2. PATHOPHYSIOLOGY OF REFLUX ESOPHAGITIS

Although the pathogenesis has been extensively studied, the primary cause of the mucosal barrier damage leading to the clinico-pathologic complications in the affected patients is still unknown. The importance of gastric acid in the progression of GERD is well-known, but the significance of other components of the refluxed material is still controversial. Most studies have found an apparent association between gastric acid reflux and esophageal inflammation. In particular, regurgitated bile can often be detected in the esophagus, and is assumed to trigger the development of Barrett's metaplasia and esophageal adenocarcinoma. However, esophagitis is a frequent finding in GERD patients with acid-suppressive therapy or even after total gastrectomy. Similarly, it has been shown that bile-induced mucosal

changes *per se* may contribute significantly to esophageal barrier lesions and the development of GERD.

Although the exact pathomechanism of GERD is not fully understood, several lines of indirect evidence suggest that a mitochondrial dysfunction may play a role in the reflux-induced esophageal mucosal responses. The hepatic mitochondria are one of the main targets of bile-induced hepatocyte damage, and the intracellular accumulation of hydrophobic bile salts during cholestasis causes hepatocyte necrosis by inducing a mitochondrial permeability transition. However, the *in vivo* effects of intraluminal bile exposure on the mitochondrial functions of the esophagus are still unclear.

Similarly, the available clinical and experimental evidence suggests that microvascular injury is a causative factor in secondary detrimental reactions, including permeability changes and bacterial or endotoxin translocation in the gastrointestinal tract. Nevertheless, the *in vivo* response of the esophageal microcirculation to acute regurgitation has not been studied.

2. AIMS

Our first goal was to create a large animal model of acute gastroesophageal reflux, where the effects of different components of the refluxed material could be observed separately. Our second aim was to obtain an insight into the initial processes leading to tissue injury during GERD. We hypothesized that mitochondrial dysfunction may be directly associated with secondary functional and structural changes in the esophageal mucosa. Accordingly, the consequences of exposure to bile with or without gastric acid, as possible luminal damaging agents, were characterized separately in our large animal model of acute GERD. In line with this goal, bile-induced morphological changes were also evaluated and compared with acid-caused structural damage.

Our next objective was to characterize the reactive microcirculatory changes in the esophageal mucosa during acute esophageal regurgitation. To this end, the mucosal microcirculatory changes were observed continuously by means of intravital videomicroscopy after a standardized biliary, acidic, or mixed challenge.

A further aim was to outline those elements which may be linked to microcirculatory alterations and could participate in the evolving mucosal dysfunction. Nitric oxide (NO) plays a central role in the maintenance of the normal resting esophageal mucosal blood flow, and it has been suggested that local changes in NO release may be critically involved in gastrointestinal inflammatory reactions. Hence, our last objective was to determine the consequences of acute reflux on the activities of the constitutive NO synthase (cNOS) and inducible NOS (iNOS) isoforms.

In the first series **(Study I)** we investigated the role of different reflux components (bile, bile + acid, and acid alone) in the mitochondrial energy turnover changes, permeability and morphological alterations on esophageal mucosa during acute reflux esophagitis.

In the second series of experiments **(Study II)** esophageal microcirculatory changes and changes in mucosal cNOS and iNOS activities were evaluated.

3. MATERIALS AND METHODS

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

3.1. SURGICAL PREPARATION

The surgical interventions were almost identical in Studies I and II. The experiments were performed on a total of 54 mongrel dogs (average weight 15.5±2 kg) under sodium pentobarbital anesthesia (30 mg kg^{-1} i.v.). Small supplementary doses of pentobarbital were administered when necessary. The animals were placed in a supine position on a heating pad for the maintenance of body temperature between 37 and 38 °C. In aseptic techniques, the left femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and for fluid and drug administration, respectively. All animals received a continuous infusion of Ringer's lactate at a rate of 10 ml kg^{-1} h^{-1} during the experiments. The esophagus was inspected by endoscopy (Olympus GIF-E) prior to the experiments to exclude major mucosal lesions. Following a collar incision, a part of the cervical esophagus with intact neurovascular connections was dissected free, and an approximately 8-

10-cm segment of the middle portion was then occluded at both ends with atraumatic clips. A plastic tube (0.5 mm i.d.) was inserted distally into the lumen and secured with a purse-string suture for the administration of test compounds. In Study I an agar electrode was placed surgically into the esophageal lumen for continuous measurement of the transmucosal potential difference. In Study II the objective of the intravital microscopic device was introduced into the middle portion of the prepared esophageal segment through a small incision.

3.2. EXPERIMENTAL PROTOCOLS

3.2.1. STUDY I.

Surgery was followed by a 20-min recovery period for cardiovascular stabilization, then 7 ml of isotonic saline (pH 7.4) was injected into the lumen of the prepared esophageal segment. Baseline variables were recorded for 30 min, then the esophagus segment was filled with test solution (7 ml) for 4 hours. At the and of the 3rd hour of the experiment sodium fluorescein (NaFl) was added into the intraluminal content and 30 min later Evans blue dye was injected i.v. At the end of the experiment, a biopsy was taken from the esophageal segment, together with a tissue sample from the aboral, intact part of the esophagus, with the freeze-clamp technique, for determination of the tissue adenosine triphosphate (ATP) concentrations. Additional biopsies were obtained for measurement of the tissue myeloperoxidase (MPO) activity and the vascular permeability index, and establishment of the severity of tissue damage.

The animals were randomized to one or other of the following groups: group 1 (n=8): saline-treated (pH 7.4) control, group 2 (n=8): bile-treated (pH 6.5), group 3 (n=5): hydrochloric acid (HCl) + bile-treated (pH 2.5), and group 4 (n=5): HCl-treated (pH 2.0). Canine bile was obtained from 3 healthy dogs before the experiments, pooled and stored at -20 °C. The intraluminal volume load was identical in all groups studied.

3.2.2. SUDY II.

Surgery was followed by a 20-min recovery period for cardiovascular stabilization, and 7 ml isotonic saline (pH 7.4) was then introduced into the esophageal segment for 30 min to determine the baseline microcirculatory

parameters. After this period, the esophagus was filled with different test solutions (7 ml) for 3 hours, meanwhile the microcirculatory parameters continuously were observed. At the end of the experiments full-thickness biopsies were taken from the esophagus for histology, and NOS and myeloperoxidase (MPO) activity measurements.

The animals were randomly allocated into 4 groups. Group 1 (n=5) received 0.9% saline (pH 7.4), group 2 (n=8) canine bile (pH 6.5), group 3 (n=8) HCl (pH 2.0), and group 4 (n=7) bile + HCl solution (pH 2.5).

3.3. MEASUREMENTS

Central venous pressure and mean arterial pressure were measured continuously with Statham P23Db transducers and registered with a computerized data-acquisition system (Haemosys 1.17; Experimetria Ltd., Budapest, Hungary). Arterial blood gases were measured with an AVL Compact 2 blood gas analyzer (AVL, Graz, Austria).

3.3.1. TRANSMUCOSAL POTENTIAL DIFFERENCE

An exploring electrode was inserted into the lumen of the esophagus, and the reference electrode was placed into the paraesophageal space. The electrodes were filled with 3% agar solution dissolved in saturated potassium chloride solution. The agar bridges were connected to a calomel electrode (Radelkis, Budapest, Hungary) and a potentiometer, and the transmucosal potential difference changes (in mV) were recorded on a continuously running polygraph.

3.3.2. EPITHELIAL PERMEABILITY INDEX

180 min after the insult, lumen-to-blood directional epithelial permeability changes were detected by the NaFl clearance method. Briefly, 40 mg of NaFl was added into the esophageal lumen. Blood samples were taken from the femoral vein in 10-min periods, and the NaFl concentration of the plasma was determined. The blood samples (2 ml) were collected in prechilled tubes containing 250 IU heparin, and immediately centrifuged at 1000 g at 4 °C for 5 min. The samples were stored at 0 °C in the dark for a maximum of 120 min. NaFl concentrations were measured with fluorescence spectrophotometer (ex: 455 nm, em: 515 nm). The lumen-to-plasma clearance of NaFl was calculated according to the following equation:

inward NaFl clearance = [NaFl concentration]serum x 100 / [NaFl concentration]test solution x volume.

3.3.3. VASCULAR PERMEABILITY INDEX

The vascular permeability index was determined by using the azo dye Evans blue, which binds rapidly to albumin and migrates with it. At 210 min in the experiments, 20 mg Evans blue ml^{-1} kg^{-1} was given i.v. in a bolus injection, and 30 min later a blood sample was taken from the femoral vein, together with tissue samples from the intact and the exposed sections of the esophagus. The mucosal layer was scraped off, rapidly placed in 5 ml of formamide, and homogenized for 1 min. The homogenate was incubated at room temperature for 20 hours and then centrifuged at 2500 g for 30 min. The absorbance of the supernatant was determined at 650 nm against a formamide blank with a UV-1601 spectrophotometer. The concentration of Evans blue was determined from a standard curve. The protein contents of the samples were determined by the procedure of Lowry *et al.* Similarly, blood samples were centrifuged at 600 g at 4 °C for 10 min and the absorbance of the 100-fold-diluted plasma was measured. Vascular permeability index was defined as the ratio of the tissue and plasma concentrations of Evans blue: Vascular permeability index=[Evans blue concentration]tissue / [Evans blue concentration]plasma x 100.

3.3.4. MYELOPEROXIDASE ENZYME ACTIVITY

The mucosal tissue MPO activity, as a marker of tissue leukocyte infiltration, was measured from mucosal biopsies by the method of Kuebler. Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 2000 g. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan), and the data were referred to the protein content.

3.3.5. ATP MEASUREMENT

A whole-thickness sample was taken from the esophagus with a Wollenberg forceps cooled in liquid nitrogen, and the tissue was stored at -70 °C. The sample was weighed, placed into a 3-fold volume of trichloroacetic acid (6% w/v),

homogenized for 1 min, and centrifuged at 5000 g. The supernatant was neutralized with saturated potassium carbonate solution. The ATP concentration was measured spectrophotometrically according to Lamprech *et al.*. The method is based on the principle that beta-nicotinamide adenine dinucleotide phosphate is used up in an enzymatic reaction catalyzed by glucose-6-phosphate dehydrogenase and hexokinase.

3.3.6. HISTOLOGY AND LIGHT MICROSCOPY

Biopsies for light microscopy were obtained from the intact and the treated parts of the esophagus of each animal. The samples were fixed in 10% phosphate-buffered formalin solution for 24 h, embedded in paraffin, sectioned (6 μm) and stained with hematoxylin-eosin. Histological analysis was performed in coded sections by one investigator. Mucosal injury was graded on the 0-100 esophageal mucosal damage score of Lanas *et al.* with the following criteria: epithelial changes (epithelial splitting, erosion and ulceration): maximal score 40; inflammation (intraepithelial leukocytes and cellular hyperplasia): maximal score 40; vascular lesions (edema, congestion and hemorrhage): maximal score 20. In Study II in parallel, the degree of damage was evaluated with the 0-16 scoring system of Geisinger *et al.*: basal cell hyperplasia: maximal score 4; intraepithelial leukocytes: maximal score 4; subepithelial leukocytes: maximal score 4; presence of ulceration: maximal score 4.

3.3.7. INTRAVITAL VIDEOMICROSCOPY

An intravital videomicroscope was used with an orthogonal polarization spectral imaging (OPS) technique (Cytoscan A/R, Cytometrics, PA, USA) to monitor microvascular perfusion changes in the esophageal mucosa. The OPS technique involves the use of reflected polarized light at 548 nm, which is the isosbestic point of oxy- and deoxyhemoglobin. Since polarization is preserved in reflection, only photons scattered from relatively deep inside the tissue contribute to the images. In this way, a virtual light source is created in the tissues, so that the vessels appear black. A 10x objective was connected to a light source with a flexible cable; in this way, the esophageal segment was not exteriorized during the study. Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis

of the videotaped images. The functional capillary density (FCD; the length of perfused nutritive capillaries per observation area; μm^{-1}), the relative vessel area (RVA; the perfused vessel area per observation area), and the red blood cell velocity (RBCV; $\mu m\ sec^{-1}$) were determined in 3 separate fields by means of a computer-assisted image analysis system (IVM Pictron®, Budapest, Hungary). All data were expressed as the means of 3 measurements at each time point.

3.3.8. NITRIC OXIDE ENZYME ACTIVITY MEASUREMENTS

Nitric oxide (NO) formation in esophageal tissues was measured by the conversion of (^{3}H)L-citrulline from (^{3}H)L-arginine according to the method of Szabó *et al.* Briefly, tissue biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM tris-(hydroxymethyl)-aminomethane-HCl 0.1 mM ethylenediaminetetraacetic acid, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride and 10 $\mu g\ ml^{-1}$ soybean trypsin inhibitor. The homogenate was centrifuged at 4 °C for 20 min at 24000 g and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100 000 MW cut-off ultrafilter). The tubes were centrifuged at 1000 g for 150 min and the concentrated supernatant was washed out from the ultrafilter with 300 µl homogenizing buffer. The samples were incubated with a cation-exchange resin (DOWEX AG 50W-X8, Na^+ form) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation and the supernatant containing the enzyme was assayed for NOS activity.

For the Ca^{2+}-dependent NOS (cNOS) activity, 50 µl enzyme extract and 100 µl reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM NADPH, 10 µM tetrahydrobiopterin, 1.5 mM $CaCl_2$, 100 U ml^{-1} calmodulin and 0.5 µCi (^{3}H)L-arginine (ICN Biomedicals, specific activity 39 Ci $mmol^{-1}$) were incubated together for 30 min at 37 °C The reaction was stopped by the addition of 1 ml ice-cold HEPES buffer (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. Measurements were performed with boiled enzyme and with the NOS inhibitor N-ω-nitro-L-arginine (3.2 mM) to determine the extent of (^{3}H)L-citrulline formation independent of the NOS activity. Ca^{2+}-independent NOS activity (iNOS) was measured without Ca-calmodulin and with EGTA (8 mM).

1 ml reaction mixture was applied to DOWEX cation-exchange resin (AG 50W-X8, Na^+ form) and eluted with 2 ml distilled water. The eluted (^{3}H)L-citrulline activity was measured with a scintillation counter. Protein contents of samples were determined by the method of Lowry *et al.*.

3.3.9. STATISTICAL ANALYSIS

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures, median values and 75th and 25th percentiles are given. p values <0.05 were considered significant.

4. RESULTS

4.1. STUDY I

The baseline values of the macrohemodynamic variables did not differ significantly in the different groups and there were no significant hemodynamic changes as compared with the baseline values during the experimental period (data not shown). In the control group, saline administration did not significantly influence the mucosal morphology as compared with that observed in the untreated part of the esophagus. In this group, the baseline mucosal permeability, the transmucosal potential difference, the ATP level and the intramucosal leukocyte accumulation remained essentially constant throughout the observation period.

Intraluminal bile resulted in an approximately 60% decrease in transmucosal potential defference (M=29.5%; 25p=18.8%; 75p=31.9%), and in significant increases in the vascular permeability index (M=0.953; 25p=0.84; 75p=1.05) and transmucosal NaFl clearance (M=4.54; 25p=4.1; 75p=5.01).

Similar changes were observed in the bile + HCl-treated group: transmucosal potential difference was significantly decreased, with parallel increases in vascular permeability index and NaFl clearance.

HCl alone induced a decrease in transmucosal potential difference and an increase in the lumen-to-blood direction mucosal permeability, similar to those observed following bile and bile + HCl administration. However, the change in vascular permeability index remained statistically nonsignificant throughout the experiments.

The leukocyte accumulation was significantly increased in the mucosa in groups 2, 3 and 4 as compared with the intact part of the esophagus, or with the saline-treated group 1. Bile, bile + HCL, or HCl administration alone resulted in a 6-fold (M=18.1; 25p=15.7; 75p=22.6), an 8-fold (M=24.6; 25p=20.4; 75p=27.8) and a 2.5-fold (M=6.65; 25p=4.9; 75p=8,05) increase in myeloperoxidase activity, respectively.

The esophageal ATP content: there were no significant differences in tissue ATP levels between the intact and treated parts of the esophagus in the saline-treated control group. Intraluminal bile and bile + HCl resulted in an approximately 40% decrease in esophageal tissue ATP level (bile: M=2.66; 25p=2.04; 75p=3.25; bile-HCl: M=2.42; 25p=2.19; 75p=3.05) by the end of the observation period. In these groups, the ATP levels were significantly reduced as compared with the saline-treated group. Administration of HCl alone did not influence the esophageal ATP content.

Tissue samples taken from the intact and saline-treated parts of the esophagus of the control group exhibited an average grade of injury of 2.57 (range of scores 0-18). Control samples from the bile, bile + HCl, and HCl-treated groups revealed the structure of the normal esophageal mucosa. In these sections, the luminal surface was lined by a continuous layer of epithelial cells.

Semiquantitative evaluation of samples from bile-treated animals revealed a significant exacerbation of the mucosal injury in all cases ($p < 0.01$) and an injury score of 62.61 (range 42-90). Moderate and severe lesions were commonly observed, with epithelial desquamation and necrosis, intraepithelial and subepithelial leukocytosis, basal cell hyperplasia and subepithelial connective tissue damage.

Less severe lesions were observed following bile + HCl treatment. In this group, mild and moderate lesions were usually seen within the same section.

Disruption of the epithelial layer, desquamation, minor necrosis, intraepithelial leukocytosis, edema of the subepithelial connective tissue and subepithelial leukocytosis were generally observed, but the deeper tissue layers were less strongly involved. The injury score was 38.66 (range 21-70).

In HCl-treated group 4, the reactive epithelial changes were different from those observed in the bile alone or bile + HCl-treated groups. The injury was apparently less severe in this environment (average score 29.7, range 18-42). The subepithelial inflammation was milder, and petechiae were rarely observed. In general, there was no evidence of deep submucosal damage, though transmural lesions could occasionally be observed.

4.2. STUDY II.

The baseline values of the macrohemodynamic variables did not differ significantly in the different groups and there were no significant hemodynamic changes as compared with the baseline values during the experimental period (data not shown). In the control group, saline administration did not significantly influence the histology scores of the mucosal morphology.

The baseline level of the capillary RBCV ranged between 430 and 470 $\mu m\ s^{-1}$ in the various groups. In the control group, the RBCV in the capillaries of the esophagus did not change during the experiments. However, the RBCV was increased significantly after the 3-h exposure to the bile or HCl-containing test solutions and average values of 607, 730 and 620 $\mu m\ s^{-1}$ were measured after bile, HCl and bile + HCl treatment, respectively.

The RVA was significantly elevated in all treated groups as compared with the sham-operated group or with the baseline. Administration of bile, HCl alone or bile + HCl resulted in a rise from of the baseline values 0.319, 0.333 and 0.312 to 0.437 (0.365; 0.473), 0.41 (0.391; 0.442) and 0.425 (0.415; 0.465), respectively.

The FCD was not influenced by luminal bile or HCl + bile treatment for 3 h. However, HCl treatment alone was followed by a significant decrease from 0.0377 μm^{-1} (0.0342; 0.040) to 0.0292 μm^{-1} (0.027; 0.0307) at the end of the experimental period.

The structural damage to the mucosa was quantified by histology using two

different scoring systems; in consequence of the highly characteristic tissue lesions, there was no significant difference between the results of the evaluation methods. The biopsy samples from the saline-treated control group exhibited an average grade of injury of 2.25 (range of scores: 0-18) on the Lanas scale, and of 0.37 (range of scores: 0-3) on the Geisinger scale. The 3-h bile exposure induced severe mucosal damage, with median values of 65 (range: 50-70) on the Lanas scale, and 12 (range 8-14) on the Geisinger scale ($p<0.01$). Deep lesions were commonly observed, with extensive epithelial loss, desquamation and necrosis, intraepithelial and subepithelial leukocytosis, basal cell hyperplasia and subepithelial connective tissue damage. In most cases, transmural inflammation, edema and vasodilatation were apparent.

In HCl-treated group 3, the reactive mucosal changes were different from those observed in the bile-treated groups. HCl administration induced severe epithelial damage with considerable basal cell hyperplasia. Petechiae and deep transmural lesions were only observed occasionally. The median value of the Lanas scores was 30 (range 19-40), and that was of the Geisinger scores 5.0 (range 3-7). Following treatment with bile + HCl, the histology revealed less severe (mild to moderate) morphological changes. Although disruption of the epithelial layer, desquamation, intra- and subepithelial inflammatory cells and subepithelial connective tissue edema were generally observed, the deeper tissue layers were less involved. The degree of injury was 33 (range: 25-50) on the Lanas scale, and 5 (range: 5-7) on the Geisinger scale.

The MPO data demonstrated that the leukocyte accumulation was significantly increased in the mucosa in groups 2, 3 and 4 as compared with saline-treated group 1. Bile, HCl alone or bile + HCl administration resulted in a 10-fold (M=50.0; 25p=29.5; 75p=76.1), 3-fold (M=15.0; 25p=11.2; 75p=17.9) and 8-fold (M=41.5; 25p=34.3; 75p=49.9) rise in MPO activity, respectively. Although the average MPO values were usually higher within the muscle, the MPO activities in the two tissue layers were not significantly different. Both bile and bile + HCl administration resulted in a 5-fold elevation, while HCl administration alone resulted in an approximately 2-fold increase in muscle MPO activity.

The changes in the esophageal cNOS and iNOS activities: the activity of

cNOS was significantly depressed after bile treatment, and this change was accompanied by a significant, approximately 5-fold increase in iNOS activity. HCl treatment did not influence the esophageal cNOS activity, while it resulted in a somewhat lower, 2.5-fold increase in iNOS activity, as compared with the value for the sham-operated group. Similarly, the cNOS activity was not influenced by bile + HCl administration for 3 hours, whereas the activity of iNOS was increased significantly, similarly as observed after bile treatment alone.

5. DISCUSSION

Despite the continuously growing body of information, the exact pathophysiology of the reflux-induced mucosal dysfunction leading to clinico-pathologic complications is still unknown. The importance of duodenal components in the progression of GERD is well known, in recent decades duodenogastro-esophageal reflux or alkaline reflux has become an autonomic entity. In a number of cases, the bile content of the regurgitated fluid has been demonstrated by a fiberoptic probe (Bilitec) in the esophagus, and it has been confirmed that a mixed reflux is more harmful than gastric juice alone.

Our *in vivo* study has provided a comparison of the separate responses to HCl and bile in the canine esophagus. The results reveal that intraluminal bile may be involved in the mechanism of esophageal ATP reduction and mucosal impairment barrier simultaneously. Bile and HCl mixed together decreased the ATP content of the exposed tissues, increased the epithelial and vascular permeability indices, evoked a rise in leukocyte accumulation, and induced severe structural alterations in the esophageal mucosa. The reduction in the mucosal ATP level was attributed exclusively to bile.

Bile acid toxicity has been repeatedly demonstrated in the hepatic tissues. It has recently been confirmed that the intrahepatic accumulation of toxic bile salts is directly connected with a mitochondrial dysfunction. Similarly, it has been shown that bile salts cause hepatocyte death by inducing mitochondrial permeability transition, Fas-dependent hepatocyte apoptosis or necrosis. Bile salts at low concentrations inhibit the activities of complexes I and III of the mitochondrial respiratory chain.

Although the disturbance of mitochondrial energy production could be an important factor in the pathomechanism of acute damage of the canine esophagus, the sequence of events is still unclear. Bile-induced ATP depletion may be an important component of the esophageal damage, but the different sensitivities of the esophageal and intestinal mucosal surfaces toward bile remains an interesting problem. Moreover, nonspecific detergent effects of bile, such as bile-induced oxidative damage, have to be considered.

At first sight, a biliary reflux alone can not evolve in GERD patients. However, HCl secretion can be completely blocked by novel proton pump inhibitor therapy. With long-term continuous proton pump inhibitor therapy, HCl suppression results in a pH > 5.0 and "iatrogenic bile reflux" may occur if a duodenogastro-esophageal reflux is also present. Further, achlorhydria or gastric acid suppressive therapy might lead to bacterial colonization in the upper gastrointestinal tract. As a result of the bacterial flora, deconjugated bile acids appear in a higher proportion, inducing severe mucosal damage in the esophagus. Thus, in patients on proton pump therapy with concomitant bile regurgitation, a "bile danger zone" of higher pH (pH > 5.0) has to be considered.

The link between gastric pH and esophageal injury has been the source of controversy. Previous studies have demonstrated a synergistic effect of pepsin and acid in mucosal damage at low pH; and these results have led the authors to suggest that gastric acid exerts an indirect effect on esophageal damage by determining peptic activity. However, it has recently been shown that the presence of pepsin did not exacerbate the severity of acid-induced esophageal injury. Similarly, it is important to note that the solubility of bile salts may be dependent on luminal pH. Although the importance of this phenomenon remains to be investigated, it seems that in our study the degree of bile induced mucosal injury was not influenced by pH.

In line with these data, there is good evidence that a 4-h bile exposure results in a process which significantly affects the permeability characteristics of the canine esophagus. The falling level of tissue ATP described here was accompanied by an increased mucosal permeability in the blood-to-lumen and lumen-to-blood directions, and by a significant decrease in transmucosal potential difference. The transmucosal

potential difference maintained across the healthy mucosa is thought to be due to asymmetric ion pumping combined with resistance to the back-diffusion of the separated charge. A decreased transmucosal potential difference is indicative of a loss of mucosal integrity and also points to a disturbed mitochondrial energy turnover. The degree of impairment of the protective mucosal barrier may range from reversible permeability changes to complete transmural necrosis. The extent of permeation across the mucosal barrier depends on the solubility and size of the molecule and on the blood flow. Equilibration of small molecules such as NaFl requires only a short period, and subtle structural changes might therefore result in rapid permeation changes in the mucosa. Although the overall results of our study suggest that the permeability alterations in the esophageal mucosa may be dependent on bile-induced ATP depletion, this possibility should be treated with caution. It is clear that the early mucosal responses to luminal damaging agents involve a multiplicity of factors, and the mechanism of chronic bile salt-induced mucosal dysfunction remains an area of active investigations. However, it is tempting to speculate that bile salts may play similar triggering roles in GERD after prolonged or intermittent bile exposure.

In our model, exposure to either HCl or bile was accompanied by a significant rise in tissue MPO activity. The contact of the neutrophil leukocyte with a chemoattractant triggers a complex metabolic event, with the production of oxygen intermediates. Once the leukocytes have entered the tissues, they might induce further endothelial and epithelial damage and interfere with the aggressive action of intraluminal agents. Although these data suggest that reflux-induced leukocyte activation and tissue accumulation might be secondary components of the pathomechanism of mucosal destruction, more direct approaches are needed to identify the roles of bile and HCl precisely in the microvascular changes and leukocyte reactions. In this regard, our results emphasize the importance of monitoring functional parameters of esophageal injuries in order to obtain valid conclusions from studies involving tissue salvage therapies.

It has been found that the intraluminal administration of HCl induces increases in esophageal permeability and MPO activity with the same efficacy as observed for

HCl mixed with bile or bile alone. Similarly, the evolved injury of the esophageal mucosa could be directly connected to the "topical" effect of HCl. On the other hand, exogenous HCl had no effect on the mucosal ATP content. It is therefore conceivable that a synergy exists between the two components, and biliary reflux may play a key role in the HCl-induced loss in mucosal integrity or significantly exacerbate the manifestation of structural mucosal damage.

Study II showed that the early microcirculatory consequences of biliary exposure appear quickly after the start of the treatment. The RBCV and the RVA were significantly increased at 90 min, while the FCD was unchanged until 180 min. The RBCV is determined primarily by the blood flow and the cross-section of the circulatory area and this suggests that significant vasodilation evolves in the mucosa. The MPO data implied that leukocytes accumulated in the mucosa, and the enzyme activity was very high in the muscle layer too. The histology also revealed the destructive effects of bile: in almost all cases, a deep, almost transmural injury was observed. These data together demonstrate that the noxious agent penetrates very deep into the esophageal wall.

NO, formed *in vivo* by the continuously active cNOS, is involved in the physiological regulation of the peripheral vascular tone. In inflammation, endotoxemia or sepsis, a distinct isoform, the inducible, Ca^{2+}-independent iNOS is activated, thereby leading to an excessive production of NO. In our study, the bile-induced microcirculatory alterations were accompanied by inverse changes in cNOS and iNOS activities. Previously, we have shown that the multifactorial ulcer-producing actions of bile are initiated by the lowering the ATP generation in the affected esophageal tissues. Although the disturbance of mitochondrial energy production could be an important factor in the acute decrease in cNOS activity in the canine esophagus, the molecular mechanism of the events is still unclear.

The effects of bile on the cNOS activity have been described in biliary fibrosis and after experimental bile-duct ligation. Recently, it has also been reported that a low cNOS and an increased iNOS activity are responsible for the exacerbation of gastric injury from luminal irritants during endotoxemia. The increased iNOS activity could be a compensatory phenomenon of cNOS inhibition or an aftermath of

inflammatory stimuli. It should be noted that a proposed cause of carcinogenesis in GERD is chronic inflammation, while others suggested that the mechanism of the mitogenic effects of bile acids may involve the N-nitrosation of glycine and taurine amides, leading to the production of carcinogenic N-nitroso amides. Because of its short half-life, the effects of NO are limited to the site of its production; thus, NO-related vascular changes mostly depend on local NO synthesis. NO plays a central role in the maintenance of the normal resting esophageal mucosal blood flow, and an increased esophageal blood flow may be a protective response against damaging gastric juice or other noxious stimuli. Similarly, the rapid induction of iNOS may in the short run protect the tissue against microbial infection. However, excess NO generation in the inflamed tissue is likely to lead directly to the formation of N-nitroso compounds, and it is tempting to speculate that the bile-induced overproduction of NO through the activation of iNOS may in the long run play a role in the mutagenic process.

HCl induced a more superficial injury as compared with bile, and the intensity and extent of leukocyte accumulation were also somewhat more moderate. The consequence of HCl exposure on the microcirculation were different too. Vasodilation and an increased flow rate were again present, but concomitantly the FCD was decreased. It has been reported that the adaptive increase in flow after HCl treatment is dependent on the paracrine effect of NO, but mast cell-derived histamine, substance P and calcitonin gene-related peptide, also play roles in this response. The acute reduction in the FCD is usually due to an imbalance in the Starling forces or intravascular cellular events influencing the circulation in the subepithelial capillary network. A decreased FCD is a characteristic of low-flow ischemia, but this condition is regularly observed with blood acidosis in both hemorrhagic shock and intentional acid infusion.

It is important that the instillation of HCl did not change the cNOS activity, which suggests that the increase in iNOS activity is a consequence of a separate, secondary inflammatory reaction. Although the amount of bioavailable NO is usually unknown, it has been shown that the iNOS mRNA expression increases within 30 min after an inflammatory challenge, and this is followed several hours later by

increased plasma levels of the NO metabolites nitrate and nitrite.

It seems that the mixture of HCl and bile induced an intermediate condition. The alkaline pH improved the microcirculatory consequences of the acid-induced damage the FCD did not change, and the detrimental effects of the bile were also mitigated as the cNOS activity was not inhibited in this case. It was recently suggested that HCl-suppressive therapy in GERD patients increases the stomach pH and that this will remove the charges on the conjugated bile acids, allowing entry into the epithelium. Our results strengthen this hypothesis and suggest that the reactive response of the esophageal mucosa to a pure biliary reflux is indeed alleviated in the presence of gastric acid.

6. SUMMARY OF NEW FINDINGS

1. Our animal model provides a useful protocol to investigate the pathophysiological and morphological consequences of acute gastroesophageal regurgitation. The experimental model allowed the first *in vivo* visualization of the canine esophageal microcirculation.

2. We have demonstrated that bile induces ATP depletion, contributes to the early mucosal permeability alterations and barrier lesions during experimental reflux, and plays an important role in intramucosal leukocyte accumulation and structural injury.

3. Our data suggest that severe bile-induced tissue injury may evolve when the pH of the refluxed material is 6.0 or above. Bile impairs the cNOS activity and enhances the iNOS activity, and may therefore induce NO overproduction-related changes in the later phases of reflux.

Medical therapy usually does not stop bile from regurgitating to the esophagus, and acid suppression is often incomplete. While acid suppressive therapy remains a cornerstone of treatment for GERD, the deleterious consequences of biliary exposure on esophageal mucosal NO synthesis could be manifested in the absence of acid. Although the extrapolation of findings of animal studies to humans could be difficult, the results underline the significance of bile in the pathogenesis of mucosal injury of GERD.

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LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE DISSERTATION

I. **Károly Szentpáli**, József Kaszaki, László Tiszlavicz, György Lázár, Ádám Balogh, Mihály Boros: Bile-induced adenosine triphosphate depletion and mucosal damage during reflux esophagitis. Scand J Gastroenterol 2001;36:459-466 (IF: **1.826**)

II. **Szentpáli Károly**, Kaszaki József, Erős Gábor, Tiszlavicz László, Paszt Attila, Lázár György, Balogh Ádám, Boros Mihály: A nyelőcső nyálkahártya mikrokeringési változásai akut kísérletes reflux alatt – Az epés komponens szerepe. Magyar Sebészet 2003;56:61-67

III. **Károly Szentpáli**, Gábor Erős, József Kaszaki, László Tiszlavicz, György Lázár, Antal Wolfárd, Ádám Balogh, Mihály Boros: Microcirculatory changes in the canine oesophageal mucosa during experimental reflux esophagitis: comparison of the effects of acid and bile. Scand J Gastroenterol 2003;38:1016-1022 (IF: **1.826**)

LIST OF ABSTRACTS RELATED TO THE SUBJECT OF THE DISSERTATION

1. **Szentpáli K**, Kaszaki J, Tiszlavicz L, Lázár Gy Jr., Balogh Á, Boros M: Epe hatására kialakuló nyelőcső nyálkahártya lézió pathomechanizmusának komplex vizsgálata kísérletes modellben. Magyar Sebészet 52: 224, 1999.

2. **Szentpáli K**, Kaszaki J, Tiszlavicz L, Lázár Gy, Balogh Á, Boros M: Bile-reflux: ATP depletion and esophageal mucosa damage during experimental esophageal reflux. Gut 45 (S5) A85: 1999.

3. **Szentpáli K.**, Tiszlavicz L., Kaszaki J., Lázár Gy., Balogh Á., Boros M.: Gastro-oesophageal reflux disease, histological aspects on experimental model. Z. Gastroenterol 38:425, 2000

4. **Szentpáli K.**, Erős G., Kaszaki J., Tiszlavicz L., Lázár Gy., Balogh Á., Boros M.: In vivo observation of microcirculatory changes during experimental gastro-esophageal reflux. Walter Brendel Presentation. Eur Surg Res 33, 107, 2001.

5. Erős G., Kaszaki J., **Szentpáli K.**, Boros M: The role of nitric oxide in the pathomechanism of biliary gastroesophageal reflux. Eur Surg Res 34 (S1):7, 2002.

6. **Szentpáli K.**, Széll M., Kaszaki J., Lázár G., Kemény L., Dobozy A., Balogh Á., Boros M.: ATP synthase and keratinocyte growth factor gene expression in experimental gastroesophageal reflux disease. Eur Surg Res 34 (S1): 80-81, 2002.

7. Erős G., Kaszaki J., **Szentpáli K.**, Boros M.: The role of nitric oxide in the pathomechanism of bile-induced esophagitis. Shock 18 (S1): 6, 2002.